Modulation of transferrin secretion by epidermal growth factor in immature rat Sertoli cells *in vitro*

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The modulation of transferrin secretion by FSH and epidermal growth factor (EGF) was studied in highly pure, primary cultures of immature rat Sertoli cells grown on a reconstituted basement membrane (Matrigel) in bicameral chambers. Sertoli cell purity was assessed by (1) morphometry, (2) alkaline phosphatase cytochemistry (a specific marker enzyme for peritubular cells) and (3) immunocytochemistry for the alpha-isoform of smooth muscle actin in contaminating peritubular cells. Results revealed a less than 0.5% peritubular cell contamination. During initial periods of culture with EGF or FSH alone or in combination, both EGF and FSH alone maintained transferrin secretion over basal values and their effects were additive. At subsequent times, EGF alone maintained transferrin secretion, but to less extent than did FSH alone, and inhibited significantly the ability of FSH to maintain transferrin secretion. The ratio of polarized transferrin secretion in response to FSH, EGF, or in combination was also examined. FSH significantly reversed the polarity of transferrin secretion, whereas EGF, although significantly reducing the ratio of apical to basal transferrin secretion, did not lead to a preferential basal secretion of transferrin. The change in the apical:basal transferrin secretion ratio, however, was not due to a reversal of the apically secreted transferrin towards a basal direction, but rather to an increase in the total basally secreted transferrin. The effects of cell density effects on transferrin secretion were then examined. At low cell density, the relative ability of EGF and FSH together to maintain transferrin secretion was greater than at high cell density, but overall transferrin secretion was greater as cell density increased. The inhibition of FSH by EGF on transferrin secretion was also density dependent: EGF significantly inhibited FSH effects at low cell density, but failed to do so at high cell density. These results suggest that regulation of transferrin secretion by Sertoli cells appears to be under dual control, involving both FSH and EGF and may provide an explanation for the mechanism by which EGF exerts a regulatory role in spermatogenesis.

Introduction

Although Sertoli cells are known to synthesize and secrete transferrin *in vitro* (Skinner and Griswold, 1980, 1982; Skinner *et al.*, 1984), the hormonal requirements regulating this synthesis and secretion are still not clear. Initial studies revealed that insulin, FSH, testosterone and retinol regulated the synthesis and release of transferrin by Sertoli cells (Skinner and Griswold, 1982) and northern analysis provided evidence that these regulatory products acted at the level of the transferrin gene in Sertoli cells (Huggenvik *et al.*, 1987; Hugly *et al.*, 1988). In contrast, later studies reported that EGF can modulate transferrin secretion in cultured Sertoli cells, but that FSH had no effect (Mather *et al.*, 1983; Perez-Infante *et al.*, 1986).

Although EGF did not appear to increase the specific transferrin mRNA, it did increase the overall mRNA levels per Sertoli cell (Heckert and Griswold, 1989). The effects of EGF on transferrin secretion in adult Sertoli cells were also shown, but in this case the presence of insulin was required (Karzai and Wright, 1992). One report, however, raised the legitimate concern that the effects of EGF on transferrin secretion by Sertoli cells *in vitro* were indirect, and mediated via contaminating peritubular cells in the cultured system (Skinner *et al.*, 1989). Moreover, several research groups clearly demonstrated that germ cells affect Sertoli cell activity (Djakiew and Dym, 1988; Janecki *et al.*, 1988; Le Magueresse and Jegou, 1988; Castellon *et al.*, 1989; Onoda and Djakiew, 1990). Hormonal and growth factor regulation of transferrin synthesis and secretion may therefore be affected significantly, depending on the number of germ cells remaining in primary cultures of Sertoli cells.

Because all of the above studies examining modulation of transferrin secretion by EGF in Sertoli cells *in vitro* have used cells grown on plastic, a substrate that fails to maintain

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functional and morphological phenotypic expression of Sertoli cells *in vivo* (Suarez-Quian *et al.*, 1984; Tung and Fritz, 1984; Hadley *et al.*, 1985, 1987; Byers *et al.*, 1986; Ailenberg *et al.*, 1988; Page *et al.*, 1990; Dym *et al.*, 1991), we examined the apparent discrepancy regarding direct effects of EGF on Sertoli cells *in vitro* using a bicameral chamber system impregnated with extracellular matrix to grow these cells (Byers *et al.*, 1986; Janecki and Steinberger, 1986; Onoda *et al.*, 1990; Suarez-Quian and Onoda, *in press*). In this culture system Sertoli cells become tall and columnar, with basally located nuclei, form characteristic Sertoli cell junctions and functional parameters of the *in vivo* cell are maintained (Byers *et al.*, 1986; Djakiew *et al.*, 1986; Janecki and Steinberger, 1986; Hadley *et al.*, 1987, 1988; Ailenberg *et al.*, 1988; Muffly and Hall, 1988; Handelsman *et al.*, 1989; Onoda *et al.*, 1990; Page *et al.*, 1990; Dym *et al.*, 1991; Kelly *et al.*, 1991; Suarez-Quian and Onoda, 1994). Furthermore, to address rigorously the purity of the Sertoli cells *in vitro* and determine the level of peritubular cell contamination, we used morphometric, histochemical and immunocytochemical methodology to determine precisely the level of peritubular cell contamination of the Sertoli cell primary cultures.

Materials and Methods

Chemicals

FSH (NIADDK-oFSH-16) was a gift from the Endocrinology Study Section of the National Institute of Health (Bethesda, MD). EGF, receptor grade, was purchased from Collaborative Research, Inc. (Lexington, MA). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO) except as specifically described below in the text.

Isolation and culture of Sertoli cells

Sertoli cells were isolated from 18-day-old Sprague-Dawley rats as described by Hadley *et al.* (1985) but as modified by Onoda *et al.* (1990) and Suarez-Quian and Onoda (*in press*). The decapsulated testes were minced into small fragments and incubated in Dulbecco's modified Eagle's medium (DMEM) (Irvine Scientific, Santa Ana, CA) containing trypsin (1.5 mg ml^{-1}) and DNase ($20 \text{ } \mu\text{g ml}^{-1}$), for 15 min at 37°C in a shaking water bath ($90 \text{ oscillations min}^{-1}$) to remove Leydig cells and other interstitial tissue. The tubule fragments (fraction 1) were washed with 10% fetal bovine serum (FBS)-DMEM, followed by a wash with DMEM alone. Subsequently, the seminiferous tubules were treated with a second enzyme solution containing collagenase (2 mg ml^{-1}) (Worthington Biochemical Corp., Freehold, NJ), hyaluronidase (2 mg ml^{-1}) and DNase ($20 \text{ } \mu\text{g ml}^{-1}$) in DMEM, for 30 min at 37°C in a shaking water bath to remove the peritubular cells. During the second enzyme digestion, fragments were agitated every 10 min by aspiration with a Pasteur pipette. To ascertain the effectiveness of the second enzyme digestion, removal of peritubular cells was monitored by examining aliquots of the digestion with an inverted phase contrast microscope. This monitoring step is crucial for yielding highly enriched Sertoli cells. That is, each batch of enzyme required that the digestion time of the second enzyme step was altered to yield pure

Sertoli cell cultures. Most peritubular cells remained in the supernatant after the sedimentation of the Sertoli cell aggregates (fraction 2). Subsequently, the supernatant was discarded unless the peritubular cells were needed for further experiments. At this point, aggregates contained approximately 50 or more Sertoli cells (fraction 3). The third enzyme solution consisted of trypsin (0.5 mg ml^{-1}), EDTA (0.1 mg ml^{-1}) and glucose (4 mmol l^{-1}) in Ca^{2+} - and Mg^{2+} - free phosphate buffered saline (PBS), and the digestion was carried out for 15 min (or less) at 37°C in a shaking water bath to obtain smaller tubules (containing 10 cells or fewer) (fraction 4). The preparations were washed with 10% FBS-DMEM followed by a wash in DMEM alone. The number of Sertoli cells per aggregate was determined by treating cells with a trypsin-versene solution (Bioproducts, Inc., Walkersville, MD) and counting the cells using a Hausser HY-Lite corpuscle counting chamber with an Olympus BH-2 microscope. The Sertoli cells were then plated at concentrations of $2 \times 10^6 \text{ cells (0.64 cm)}^{-2}$ per well on Matrigel (Collaborative Research Inc., Lexington, MA) coated filters in bicameral chambers (Millicell-HA: Millipore Corp., Bedford, MA) and cultured with serum-free defined medium (SFDM) (Hadley *et al.*, 1985) containing FSH (100 ng ml^{-1}) and EGF (10 ng ml^{-1}) for 5 days at 32°C in a mixture of 5% CO_2 :95% air until the formation of monolayers. The Matrigel was prepared by diluting the stock solution purchased from Collaborative Research at a ratio of 1:5 with distilled H_2O . Approximately $50\text{--}75 \text{ } \mu\text{l}$ of this solution was added to each well. On the third day after plating, Sertoli cells were rinsed for 2.5 min in sterile $20 \text{ mmol tris-HCl l}^{-1}$ buffer, pH 7.5, to lyse the remaining germ cells that adhere to the monolayers (Galdieri *et al.*, 1981), and the lysis buffer was then replaced with fresh SFDM containing FSH and EGF. Medium was replaced every 2 days thereafter with fresh medium and the cultures were continued until use in the experiments. Sertoli cell aggregates after the second or third enzymatic digestion were sometimes plated onto glass coverslips or Matrigel impregnated bicameral chambers to analyse the level of peritubular cell contamination. These cells were subsequently processed with hypotonic buffer and cultured as previously described.

SFDM consisted of DMEM supplemented with $100 \text{ U penicillin ml}^{-1}$, $0.25 \text{ } \mu\text{g fungizone ml}^{-1}$, $100 \text{ } \mu\text{g streptomycin ml}^{-1}$, $2 \text{ } \mu\text{g insulin ml}^{-1}$, $5 \text{ } \mu\text{g human transferrin ml}^{-1}$, $50 \text{ ng vitamin A ml}^{-1}$, $200 \text{ ng vitamin E ml}^{-1}$, $10^{-9} \text{ mol hydrocortisone l}^{-1}$, $10^{-7} \text{ mol testosterone l}^{-1}$, $10^{-8} \text{ mol oestradiol l}^{-1}$, $2 \text{ mmol glutamine l}^{-1}$, $5 \text{ ng sodium selenate ml}^{-1}$, $1 \text{ mmol sodium pyruvate l}^{-1}$, $22 \text{ mmol sodium lactate l}^{-1}$ and $3 \text{ } \mu\text{g cytosine arabinoside ml}^{-1}$.

Morphological studies

Light microscopy. During the preparation of Sertoli cell cultures, aliquots of the Sertoli cell aggregates from the different digestion stages described above were obtained, fixed by immersion in 5% glutaraldehyde in $0.2 \text{ mol s-Collidine buffer l}^{-1}$, pH 7.4, for 2 h at room temperature and smears were prepared on glass slides. Aliquots of fractions 1-4 were examined with a phase microscope fitted with a $\times 40$ planapochromat phase objective. In addition, the tubule-cell aggregates were rinsed three times in buffer and post-fixed for

1 h in potassium ferrocyanide-reduced OsO_4 at room temperature. The isolated tubule-cell aggregates were then rinsed in buffer and dehydrated through a graded series of alcohols, followed by propylene oxide. The specimens were infiltrated with an 1:1 mixture of propylene oxide and Epon, and embedded in Epon. Sertoli cells growing *in vitro* on the bicameral chambers were processed similarly and embedded in Epon. For examination by light microscope, one micron sections were cut, mounted on slides and stained with toluidine blue. Sections were examined with a Zeiss Axiophot microscope using a $\times 63$ planapo, 1.4 N.A. objective. Photographs were taken with Kodak 4162 film and processed following the manufacturer's instructions.

Cytochemistry and immunocytochemistry

Identification of peritubular cells with alkaline phosphatase and Sertoli cells with Oil Red O cytochemistry. The presence of peritubular cells contaminating Sertoli cell monolayers *in vitro* can be ascertained by performing alkaline phosphatase cytochemistry, followed by staining Sertoli cell neutral lipids with Oil Red O as described by Chapin *et al.* (1987). Briefly, cell aggregates from fraction 3 or fraction 4 cells were plated onto glass coverslips and cultured for 5 days as previously described. On day 3 of culture, cell monolayers were treated with hypotonic buffer to lyse the contaminating germ cells as previously described. The cells were then fixed with 4% formaldehyde for 10 min at room temperature and rinsed two times with 0.25 mol 2-amino-2-methyl-1-propanol l^{-1} (AMP) at pH 8.9, containing 1.25 mmol $\text{MgCl}_2 \text{l}^{-1}$. Peritubular cells were identified by incubating the cells for 10 min in 2 mmol Fast Blue RR l^{-1} in 0.125 mol AMP l^{-1} –0.625 mol $\text{MgCl}_2 \text{l}^{-1}$ –2 mol naphthol AS-BI phosphoric acid l^{-1} at room temperature until a colour reaction developed, followed by two washes with AMP buffer and one rinse with 100% propylene glycol. Sertoli cells were identified in the same coverslip by staining with 0.5% Oil Red O (w/v in propylene glycol) for 45 min at 32°C. After one rinse with 85% propylene glycol and two washes with AMP buffer, cell nuclei were counterstained with haematoxylin and coverslips mounted on glass slides.

Immunocytochemical staining of alpha-actin in peritubular cells. The presence of peritubular cells *in vitro*, enriched for in the cell suspension supernatant of fraction 2, or as contaminants in the Sertoli cell aggregates of fraction 4, can also be ascertained by immunostaining for alpha-actin as described by Tung and Fritz (1990). This technique uses a commercially available monoclonal antibody (Mannheim-Boehringer, Indianapolis, IN) raised against the alpha-isoform of actin present in smooth muscle cells. Briefly, cells are plated on glass coverslips as described above, allowed to grow for 5 days and then fixed for 10 min with ice-cold methanol, followed by an extensive rinse in PBS, at pH 7.4. Nonspecific sites were blocked with 3% ovalbumin, followed by incubation for 30 min at 37°C in a moist chamber with the monoclonal antibody diluted 1:200. The cells were then rinsed extensively with PBS and the second antibody (Rhodamine-goat anti-mouse) diluted 1:400 was added and the cells were incubated for 1 h at 37°C in a moist chamber. After an extensive wash in PBS, the coverslips were

mounted on glass and examined by epifluorescence microscopy and phase microscopy.

Determination of Sertoli cell purity

The level of peritubular cell contamination of Sertoli cell cultures prepared as described above was determined as follows. (1) Sertoli cells from fractions 2, 3 and 4 were plated onto Matrigel impregnated bicameral chambers and processed for embedding in plastic, 1 μm sections were cut (such that cells appeared perpendicular to their plane of growth) and the cells were examined with a light microscope. One thousand cells were counted from 1 μm plastic sections prepared from three different bicameral chambers. The cell counts were performed from profiles of sections photographed with a Zeiss microscope fitted with a $\times 63$ planapo objective. (2) Sertoli cells from fractions 3 and 4 were plated onto glass coverslips and the level of peritubular cell contamination determined by staining for alkaline phosphatase. Three coverslips from three independent cell preparation experiments were processed for alkaline phosphatase cytochemistry, photographed and 1000 cells counted from each coverslip. (3) The level of peritubular cell contamination in Sertoli cells was also determined by immunostaining for smooth muscle alpha-actin in cells plated onto glass coverslips. Over one thousand cells from randomly collected fields from three coverslips of three independent cell preparation experiments were photographed, both with epifluorescence and phase illumination, and the number of cells immunostained for alpha-actin was determined by comparing the fluorescence and phase profiles.

Determination of transferrin

The amount of transferrin secreted by Sertoli cells into the culture media was determined by enzyme-linked immunosorbent assay (ELISA) as described previously using Bio-Rad's alkaline phosphatase kits (Djakiew and Dym, 1988). Sertoli cells were precultured for 5 days in SFDM containing EGF and FSH as described above and media were replaced with fresh media (0.5 ml), containing either EGF, FSH or EGF plus FSH or no additions, in both apical and basal culture chambers. Media were collected and replaced by fresh media to assay for transferrin at 48 and 96 h. The antibody (Cooper Biomedical, Malvern, PA) used in the ELISA was directed against rat serum transferrin. The sensitivity of the assay was at least 3 ng per assay well, and the detectable crossreactivity in fresh SFDM, which contained human transferrin, was less than 3 ng per assay well (Onoda *et al.*, 1990).

Statistical analysis

Statistical analyses were performed using either Student's *t* test for comparison of two groups or ANOVA when comparing different groups within an experiment. The ANOVA analysis was performed using a computer program (INSTAT, version 1.13) manufactured by Graphpad (San Diego, CA). Differences were considered significant when $P < 0.05$.

Table 1. Percentage of peritubular cell and germ cell contamination of rat Sertoli cell primary cultures

Assay	Fraction 3		Fraction 4	
	Peritubular cells	Germ cells	Peritubular cells	Germ cells
Morphometry	2	0	0	0
Alkaline phosphatase cytochemistry	2	—	0	—
Alpha-actin immunocytochemistry	4	—	0.5	—

Peritubular cell contamination was determined using three different assays, whereas germ cells were measured only with morphometry.

Results

Purity of Sertoli cell cultures

The principal contaminants of primary cultures of Sertoli cell monolayers are peritubular cells. Thus, to determine precisely the effects of a peptide hormone or growth factor on Sertoli cells *in vitro*, the purity of the Sertoli cells must be ascertained.

The level of peritubular cell contamination of the Sertoli cell monolayer was assessed using three different methods (morphometry, histochemistry and immunocytochemistry) (Table 1). Neither peritubular cells nor germ cells were detected in the Sertoli cell monolayers, plated from fraction 4, when cells were prepared for conventional observation with a light microscope. Similarly, alkaline phosphatase cytochemistry demonstrated that peritubular cells were not detectable in fraction 4 of the Sertoli cell cultures after the third enzymatic digestion of the isolation procedure. In contrast, alpha-actin immunostaining of the same preparation demonstrated a 0.5% peritubular cell contamination of the Sertoli cell monolayers. In cells plated from fraction 3, however, 2% and 4% peritubular cell contamination measured by cytochemistry or immunocytochemistry, respectively, were detected. Since neither an enzyme nor specific antibody were available to label germ cells, this level of contamination could only be measured by morphometric analysis, that is by directly counting cells growing in the bicameral chambers.

Typical preparations of Sertoli cell and peritubular cell cultures after the different stages of isolation are shown (Figs 1 and 2). Even though peritubular cells were not detected by examination with light microscope of plastic sections in fraction 3, alkaline phosphatase histochemistry revealed their presence, which consisted of (approximately) less than 2% of the total cell population (Fig. 1a). In contrast, alkaline phosphate positive cells were not observed in Sertoli cell cultures plated from fraction 4 in preparations of cells examined in this study (Fig. 1b). A sample of the level of peritubular cell contamination of Sertoli cell monolayers plated from fraction 4, revealed by smooth muscle alpha-actin immunostaining, is shown (Fig. 2). For comparison, the enriched presence of peritubular cells in the cell suspension supernatant from fraction 2 are demonstrated (Fig. 2a). That the cells immunostained are indeed peritubular cells and not Sertoli cells was ascertained by the immunostaining pattern. The pattern observed is that of stress fibres, a characteristic of peritubular cells *in vitro* and not of Sertoli cells.

Sertoli cells plated onto Matrigel and cultured in bicameral chambers resemble closely the cells *in vivo*. Under these conditions, characteristic morphological features of *in vivo* Sertoli cells were readily observed in the Sertoli cells *in vitro* at the level of resolution provided by the light microscope. These parameters included a basally located, invaginated nucleus exhibiting a prominent nucleolus and sparse heterochromatin, and a large amount of apical, supranuclear cytoplasm; these observations were consistent with our previous report (Onoda *et al.*, 1990). These distinct morphological features distinguished the Sertoli cells from peritubular cells *in vitro*, which were characterized by their elongated morphology and densely staining cytoplasm. The presence of peritubular cells contaminating the Sertoli cell monolayers was also readily observed in similar profiles (Fig. 3a, b) of cells plated from fractions 2 or 3. The peritubular cells were always observed to be localized beneath the Sertoli cells *in vitro*, as in the *in vivo* situation. Although peritubular cells were observed in Sertoli cell cultures plated from fractions 2 or 3, they were not observed in cultures of cells plated from fraction 4 (Fig. 3c). Furthermore, when Sertoli cell aggregates were plated in the bicameral chambers after the second enzymatic digestion, these relatively larger cell aggregates often formed multi-layers of Sertoli cells in cultured chambers (Fig. 3a).

Effect of FSH and EGF on transferrin secretion by Sertoli cells

Highly enriched Sertoli cells were plated in bicameral chambers and precultured for 5 days with complete SFDM containing FSH and EGF to determine the effects of EGF on transferrin secretion by Sertoli cells *in vitro*. The media were removed, replaced with fresh SFDM, with or without FSH and EGF, and collected at intervals of 2 days up to 4 days (Fig. 4). During the 4 days of the experiment, the secretion of transferrin by cells grown in the absence of FSH and EGF (SFDM control) decreased with time. Continuous administration of FSH to Sertoli cells significantly maintained the secretion of transferrin over SFDM control values and this maintenance was retained for the two collection periods. EGF alone could also significantly maintain the secretion of transferrin during the collection periods (48 and 96 h) of the experiment when compared to SFDM control values, although the ability of EGF to maintain the secretory activity was less during the second collection period (2–4 days) when compared with the first period (day 0–2). Maximal transferrin secretion by Sertoli cells was detected during the initial 2 day collection period in cells

Fig. 1. Identification of peritubular and Sertoli cells by a cytochemical method. (a) Cell aggregates from fraction 3 plated onto glass coverslips, (b) cell aggregates from fraction 4. Alkaline phosphatase activity in peritubular cells (p) is observed by the deposition of the blue reaction product, and the neutral lipid droplet (short arrows) in Sertoli cells (s) is revealed by staining with Oil Red O. g: Germ cell. Note that Sertoli cell nuclei contain only one nucleolus (long arrows), a characteristic of these cells. Scale bars represent 22.5 μm .

maintained in the constant presence of both FSH and EGF. However, during the subsequent collection period, EGF decreased significantly the ability of FSH to maintain transferrin secretion by Sertoli cells (Fig. 4b).

The polarized secretion of transferrin in response to FSH, EGF or the addition of both was also studied (Table 2). During both collection periods the ratio of apical to basal transferrin secretion was highest when Sertoli cells were grown in SFDM only. This preferential apical secretion ratio was significantly reversed by the addition of FSH to SFDM. In addition, EGF diminished significantly the ratio of apical to

basal transferrin secretion, although it did not reverse the apical to basal ratio as did addition of FSH to SFDM. Furthermore, the results indicated that the decrease in the ratio of apical to basal secretion was due to an increase in the basal secretion of transferrin, rather than to a decrease in the apical secretion, as both EGF and FSH alone could maintain higher total transferrin secretion by Sertoli cells than by SFDM alone (Fig. 4).

The ability of EGF and FSH together to maintain Sertoli cell transferrin secretion as a function of cell density was then examined. The maximum amount of transferrin secretion

Fig. 2. Identification of peritubular cells by smooth muscle alpha-actin immunostaining. The level of peritubular cell contamination in Sertoli cell monolayers plated onto glass coverslips was determined by immunostaining for smooth muscle alpha-actin. (a) The cell suspension supernatant from fraction 2, plated onto glass coverslips, was enriched for peritubular cells that immunostain positive for alpha-actin (*) on stress-fibres. In the corresponding phase image (b) note that Sertoli cells (s) do not immunostain with this antibody. (c) and (d) The one peritubular cell (arrow) was identified by a positive reaction in Sertoli cell culture obtained from fraction 4. Scale bars represent 20 μ m ((a) and (b)) and 50 μ m ((c) and (d)).

measured during the initial collection period occurred when both EGF and FSH were included in the media (Fig. 4). When the same experiment was repeated but the cell density was altered, the ability of FSH and EGF in combination to maintain Sertoli cell transferrin production at greater levels than FSH stimulation alone was observed only at low cell density (Fig. 5a). Similarly, the inhibitory effect of EGF on FSH Sertoli cell transferrin maintenance during the second collection period also depended on cell density: as cell density increased, the effects of EGF decreased (Fig. 5b).

Discussion

The role of EGF in the modulation of transferrin secretion by primary cultures of Sertoli cells obtained from immature animals was examined using an *in vitro* system that favours the *in vivo* phenotypic expression of these cells. Three methods were used to monitor the purity of the Sertoli cell cultures and at most, less than a 0.5% level of contamination was observed. Thus, use of these tissue preparation techniques and culture conditions rendered extremely pure Sertoli cells *in vitro*.

Fig. 3. Morphology of Sertoli and peritubular cells grown in bicameral chambers impregnated with Matrigel. The distinct morphology of Sertoli cells (s) distinguished this cell type from the peritubular cells (arrowheads) when the two were present in the same profile. (a) A profile of cell aggregates obtained in fraction 2; (b) cell aggregates obtained in fraction 3 and (c) cell aggregates obtained in fraction 4. Profiles such as these were used to calculate the level of peritubular cell contamination in Sertoli cell monolayers. ECM: extracellular matrix. Scale bars represent 10 μm : (a) and (c); 15 μm : (b).

It is possible to argue, however, that assessing Sertoli cell purity by either alpha-actin immunostaining or alkaline phosphatase cytochemistry of cells plated onto glass coverslips are invalid methods for determining purity of Sertoli cells growing on bicameral chambers impregnated with Matrigel. For example, the cells plated on glass coverslips may express different plating efficiencies, viabilities or rates of division from those of cells plated onto the Matrigel impregnated bicameral chambers. Several arguments can dispel this criticism. (1) The level of purity of Sertoli cells obtained for cells growing on the

two different substrata were almost identical. (2) Plating efficiency for peritubular cells between glass and bicameral chambers does not seem to differ (Hadley *et al.*, 1985; Byers *et al.*, 1986; Suarez-Quian and Onoda, in press). (3) All the cells were grown with cytosine arabinoside, which prevents cell division. Thus, assaying purity of cells growing on the glass coverslips measured the level of cell contamination which was plated onto the Matrigel substrate and serves to validate our isolation scheme for enriching the purity of the Sertoli cells used for plating. (4) In several studies, Sertoli cell viability was

Fig. 4. Effect of FSH and epidermal growth factor (EGF) alone and in combination on transferrin secretion by Sertoli cells cultured in a bicameral chamber system. Sertoli cells (2×10^6 cell in $(0.64 \text{ cm})^{-2}$ per well) were plated and precultured with serum-free defined medium (SFDM) (plus EGF and FSH) for 5 days, then media were replaced with fresh SFDM containing either FSH (100 ng ml^{-1}), EGF (10 ng ml^{-1}), or both. Conditioned media were collected from the apical and basal chambers separately and replaced with fresh media every 2 days for 4 days. Each value represents mean $\pm$ SEM obtained from four wells, and is representative of three independent experiments (a) 0–48 h, (b) 48–96 h. *Significant difference ($P < 0.01$) between FSH, EGF and FSH + EGF treated cells and SFDM control cells. **Significant difference ($P < 0.05$) between SFDM + FSH and SFDM + FSH + EGF.

Table 2. Polarity of transferrin secretion by rat Sertoli cells cultured in bicameral chambers

Treatment	Ratio of transferrin secretion (Apical:basal)	
	0–48 h	48–96 h
SFDM	1.57 ± 0.29	1.58 ± 0.10
SFDM + FSH	$0.84 \pm 0.05^{**}$	$0.89 \pm 0.04^{**}$
SFDM + EGF	$1.07 \pm 0.12^{*}$	1.24 ± 0.15
SFDM + FSH + EGF	$0.84 \pm 0.05^{**}$	$0.92 \pm 0.06^{**}$

Values represent means $\pm$ SEM obtained from the same experiment as shown in Fig. 4.

*Significant difference between SFDM and EGF treatment group ($P < 0.05$).

SFDM: serum-free defined medium; EGF: epidermal growth factor.

**Significant difference between SFDM and other treatment groups ($P < 0.01$).

shown not to differ between cells grown on glass coverslips versus Matrigel (Hadley *et al.*, 1987; Dym *et al.*, 1991; Suarez-Quian and Onoda, in press). Rather, it was the phenotype of cells that differed.

Using this culture system, we demonstrated that both FSH and EGF alone can maintain transferrin secretion by Sertoli cells during the initial collection period and that maximal maintenance was achieved with a combination of both. The ability of EGF to maintain Sertoli cell transferrin secretion indepen-

dently decreased with time in culture. In addition, we demonstrated that continuous stimulation of Sertoli cells with EGF caused a significant decrease in the ability of FSH to maintain transferrin secretion. However, the decreased EGF responsiveness was not due to an inability of the EGF to encounter its receptor. Both the apical and basal media bathing the Sertoli cells contained EGF. Furthermore, we previously demonstrated that when EGF was administered to the basal compartment of confluent, polarized Sertoli cells growing in bicameral chambers, it diffused freely across the millipore support on which the Sertoli cells were placed, was internalized via a receptor-mediated endocytosis mechanism, and was degraded (Suarez-Quian *et al.*, 1989). Spaliviero and Handelsman (1991) demonstrated that both FSH and EGF can reverse the polarity of transferrin secretion by Sertoli cells, from a more apical to a more basal direction. This reversal in polarity was due to an enhanced secretion of transferrin into the basal compartment and does not occur at the expense of apical secretion. Our results confirmed these observations for FSH, but differ slightly for EGF in that we did not observe as great a polarity shift in transferrin secretion as reported by Spaliviero and Handelsman (1991). Methodological differences in the Sertoli cell culture conditions between the two groups, however, may account for these differences. In the earlier work, for example, concentrations of EGF added to the Sertoli cells were higher (50 ng ml^{-1} versus 10 ng ml^{-1}), the cells were maintained at a higher temperature (37°C versus 32°C), cell densities in general were higher, and the duration of the experiments was different.

The evidence presented herein that EGF alone can maintain transferrin secretion by Sertoli cells and that EGF modulates FSH stimulation of transferrin secretion may help explain previous discrepancies with respect to the regulation of transferrin secretion by Sertoli cells. Different culture conditions, media compositions or times at which cells were assayed by different investigators undoubtedly led to these discrepancies. In this context, also, it is important to note that a consensus has emerged from numerous studies that the substratum on which cells grow modulates profoundly the functional activity of cells *in vitro* (reviewed in Hay, 1983; McDonald, 1987). In the case of FSH regulation of Sertoli cell activity, for example, Dym *et al.* (1991) showed that Sertoli cells grown on Matrigel responded to physiological concentrations of FSH ($25\text{--}50 \text{ ng ml}^{-1}$), whereas pharmacological concentrations of FSH (500 ng ml^{-1}) were required to raise cAMP concentrations of cells grown on plastic culture dishes.

Another consideration described herein that may have led to discrepancies regarding the hormonal control of transferrin secretion by Sertoli cells is cell density. The EGF–EGF-receptor system is known to undergo receptor downregulation as a function of an increase in cell density, in the absence of either exogenously added ligand or via an autocrine stimulation (Holley *et al.*, 1977; Rizzino *et al.*, 1988; Lichtner and Schirmacher, 1990; Suarez-Quian and Byers, 1993). This phenomenon, termed density-induced receptor downregulation, has been observed to occur in several growth-factor–growth-factor-receptor systems in different cells and is believed to be correlated with a significant decrease in EGF responsiveness (Holley *et al.*, 1977; Rizzino *et al.*, 1988, 1990). In the present study, the inhibitory effect of EGF on FSH maintenance of transferrin secretion was clearly dependent on cell density.

Fig. 5. Effects of EGF and FSH on transferrin secretion as a function of cell density. Sertoli cells cultured for 5 days in serum-free defined media (SFDM), then treated with either FSH or FSH + epidermal growth factor (EGF) and total transferrin secreted during (a) the first 48 h, or (b) the subsequent 48 h was measured by enzyme-linked immunosorbent assay. In (a) as cell density increases up to 2×10^6 cells per well, total transferrin secretion also increases, but the relative difference between ($\square$) SFDM + FSH and ($\square$) SFDM + FSH + EGF was greater at low cell density. In (b), the inhibitory effects of EGF on the effects of FSH are also greater at low than at high cell density. Each value represents mean $\pm$ SEM obtained from four wells, and is representative of three independent experiments. * $P < 0.05$; ** $P < 0.01$.

Thus, besides the substratum, the effects of cell density may also account in part for the reported discrepancies in transferrin regulation by hormones. Our studies, however, do not support the suggestion that the effects of EGF on Sertoli cells are mediated by contaminating peritubular cells in the culture (Skinner *et al.*, 1989) and suggest that the EGF receptor present in Sertoli cells (Suarez-Quian *et al.*, 1989; Suarez-Quian and Niklinski, 1990; Foresta *et al.*, 1991; Radhakrishnan and Suarez-Quian, 1992) is a functional receptor.

The role of EGF in spermatogenesis has been the subject of an intensive investigation since Tsutsumi *et al.* (1986) showed that ablation of the submandibular gland rendered male mice infertile. Significantly, the sialoadenectomy effects were reversed by daily administration of EGF and not of other growth factors present in the submandibular gland. The effects of ablation of the submandibular gland on the testis have been corroborated in part by several investigators (Russell *et al.*, 1990; Suarez-Quian *et al.*, 1990; Seethalakshmi *et al.*, 1991), although the original suggestion that the submandibular gland may exert an effect on the testis was made by Katagiri and Higashijo (1940) and Suzuki (1957), working on immature rats and guinea pigs, respectively, who demonstrated that sialoadenectomy has deleterious effects on the testis. That EGF is likely to play a crucial role in spermatogenesis has also been substantiated in the streptozotocin-induced diabetic mouse model. In these mice, concentrations of EGF in plasma are significantly lower than in controls and oligozoospermia ensues, an effect which can be reversed by daily treatment with EGF (Noguchi *et al.*, 1990). Taken together, these studies support the hypothesis that EGF plays a major role

in regulating spermatogenesis, although the mechanism of action remains obscure. Results of the study reported here suggest that one means by which EGF can exercise control of spermatogenesis is by modulation of Sertoli cell transferrin production.

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